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# Iron Homeostasis: Recently Identified Proteins Provide Insight into Novel Control Mechanisms*

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**Iron is an essential nutrient required for a variety of biochemical processes. It is a vital component of the heme in hemoglobin, myoglobin, and cytochromes and is also an essential cofactor for non-heme enzymes such as ribonucleotide reductase, the limiting enzyme for DNA synthesis. When in excess, iron is toxic because it generates superoxide anions and hydroxyl radicals that react readily with biological molecules, including proteins, lipids, and DNA. As a result, humans possess elegant control mechanisms to maintain iron homeostasis by coordinately regulating iron absorption, iron recycling, and mobilization of stored iron. Disruption of these processes causes either iron-deficient anemia or iron overload disorders. In this minireview, we focus on the roles of recently identified proteins in the regulation of iron homeostasis.**

## Iron Absorption and Recycling

*Iron Absorption*—Adults absorb ~1–2 mg of iron/day from the diet to compensate for daily iron loss due to the sloughing of epithelial cells, blood loss, and sweat. Most diets contain two different forms of iron, inorganic non-heme iron in vegetables and grains and heme iron (ferrous iron protoporphyrin IX) in red meat. Iron traverses both the apical and basolateral membranes of absorptive epithelial cells to reach the blood, where it is incorporated into Tf,[2] the major iron transport protein. At least two different sets of transporters are used in this process. Non-heme iron is transported by DMT1 (divalent metal transporter 1), the intestinal iron importer. Dietary non-heme iron exists mainly as $Fe^{3+}$ and has to be reduced prior to transport. DcytB, a reductase whose expression is induced by iron deficiency, is localized in the apical membrane of intestinal enterocytes and is a major but most likely not the only reductase. The transporter responsible for heme uptake remains controversial (1–3).

Cytosolic iron in intestinal enterocytes can be either stored in the cytosolic iron storage molecule, ferritin, or exported into plasma by the basolateral iron exporter, FPN. FPN is most likely the only cellular iron exporter in the duodenal mucosa as well as in macrophages, hepatocytes, and the syncytial trophoblasts of the placenta because targeted deletion of both FPN alleles in mice is embryonic lethal. The export of iron by FPN depends on the multicopper oxidase, Cp, in the circulation and hephaestin on the basolateral membrane of enterocytes, which convert $Fe^{2+}$ to $Fe^{3+}$ for incorporation of iron into Tf. Tf-bound iron is the major iron source for most tissues.

*Iron Distribution in the Body*—Adults have a total of 3–5 g of iron. Approximately 65–75% is found in the hemoglobin of erythrocytes in the form of heme. The liver stores 10–20% in the form of ferritin, which can be mobilized easily when needed. About 3–4% of the body's iron is in heme-bound myoglobin in striated muscle. The rest is distributed in other tissues. Under physiological conditions, ~25 mg of iron/day is consumed by immature erythrocytes in bone marrow for heme biosynthesis.

*Iron Recycling within the Body*—Macrophages in the liver and spleen are responsible for the recycling of heme iron from senescent erythrocytes. The hemoglobin-derived heme is catabolized by the cytosolic heme oxygenase-1 to release iron, and the iron is subsequently exported into the circulation by FPN. In addition, heme can also be exported directly into the circulation via the heme exporter, FLVCR (feline leukemia virus subgroup C receptor), on macrophage plasma membranes. A recent study demonstrated that FLVCR also plays a critical role in the export of excess heme from immature erythrocytes and hepatocytes (4). Plasma heme is scavenged and transported by hemopexin to hepatocytes for degradation. Iron recycling from senescent erythrocytes in macrophages constitutes the major iron supply for hemoglobin synthesis.

## Cellular Iron Sensing and Regulation

The majority of cells obtain their iron requirements by Tf-mediated iron uptake via TfR1. TfR1 is internalized into endosomes that are acidified, facilitating the release of iron from Tf (5, 6). The iron is reduced by a recently identified ferrireductase, Steap3, and transported across the vesicle membrane for utilization within the cell and/or storage (7). DMT1 is the transporter in immature red blood cells (8). Iron uptake is roughly proportional to the number of TfRs on the cell surface. Regulation of TfR1 is achieved via IRPs and mRNA stem-loop structures, IREs, which have been reviewed extensively (9, 10). The IREs in mRNA of TfR1 negatively regulate the stability of TfR1 mRNA when cytosolic iron levels are high. Under low iron conditions, the IRPs bind to the IREs, where they stabilize TfR1 mRNA. The double knock-out of IRP1 and IRP2 is embryonic lethal. The double knock-out of these genes in the intestine results in the death of intestinal epithelial cells, presumably by iron depletion (11), underscoring the importance of these proteins.

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[2] The abbreviations used are: Tf, transferrin; FPN, ferroportin; Cp, ceruloplasmin; TfR, Tf receptor; IRP, iron-responsive protein; IRE, iron-regulated element; HJV, hemojuvelin; BMP, bone morphogenic protein; HFE, hereditary hemochromatosis protein; TGF-$\beta$, transforming growth factor-$\beta$; HH, hereditary hemochromatosis; HIF, hypoxia-inducible factor.

### Liver as the Central Iron Regulatory Organ

Hepcidin, a peptide synthesized by the liver hepatocytes, plays a major role in regulating iron homeostasis in the body (9, 10). The mature form is 25 amino acids with four intersubunit disulfide bonds. The massive iron overload found in hepcidin knock-out mice suggests that hepcidin is an iron stores regulator involved in communication of body iron status to the intestine (12). In contrast, mice engineered to overproduce hepcidin are severely anemic (13). The discovery that a hepatic adenoma overexpressing hepcidin results in anemia and that the anemia is resolved upon removal of the tumor confirms the relationship between hepcidin expression and inhibition of iron uptake by the intestine (14). Studies have demonstrated that hepcidin binds FPN, which results in the internalization and degradation of FPN (15). Hepcidin therefore functions to decrease serum iron levels by blocking iron absorption from the intestine, iron recycling from macrophages, and mobilization of stored iron from liver hepatocytes.

The liver plays a major role in iron homeostasis in the body in addition to secreting hepcidin. Liver macrophages take up senescent red blood cells and hemoglobin through the hemoglobin-haptoglobin receptor (CD163), salvage the iron released from hemoglobin, and secrete the iron as $Fe^{2+}$ via FPN. Hepatocytes synthesize both Tf and Cp. Cp facilitates the efflux of iron from cells as well as the loading of iron into Tf (16, 17). Hepatocytes take up Tf through TfR1 and the more recently identified TfR2 (18). They also take up other forms of non-Tf-bound iron, including heme via the heme hemopexin receptor (19), and are capable of storing large quantities of iron in ferritin and hemosiderin, a breakdown product of ferritin. Thus, the liver and, in particular, the hepatocyte are thought to sense and reflect body iron stores (20).

### Iron Sensing and Regulation of Hepcidin Expression

Humans possess elegant mechanisms to maintain iron homeostasis by modulating the expression of hepatic hepcidin. HJV, BMPs, TfR2, HFE, and Tf are critical to this process. Hepcidin expression is also regulated by erythroid factors, hypoxia, and inflammation, regardless of body iron levels.

*HJV and BMPs*—HJV is a recently identified protein encoded by the gene *HFE2*. Both clinical and animal studies demonstrate that it plays a pivotal role in iron homeostasis. Homozygous or compound heterozygous mutations in *HFE2* are responsible for the onset of the majority of juvenile hemochromatosis. Disruption of both *Hfe2* alleles in mice ($Hjv^{-/-}$) results in a marked increase in iron deposition in the liver, pancreas, and heart (21–23). The severely suppressed expression of hepcidin, detected in juvenile hemochromatosis patients and in $Hjv^{-/-}$ mice, indicates that HJV plays a central role in the regulation of hepatic hepcidin expression.

HJV is highly expressed in skeletal muscle and heart and at lower levels in liver and binds both BMPs and neogenin (24–26). Recent studies show that HJV is a co-receptor for BMP2, BMP4, BMP5, and BMP6 and that it increases hepatic hepcidin expression via enhancing BMP signaling (24, 27). BMPs are a subfamily of cytokines that belong to the TGF-$\beta$ superfamily. The BMP subfamily signals through one set of receptor-activated SMADs (SMAD1, SMAD5, and SMAD8). The receptor-activated SMADs form heteromeric complexes with SMAD4, the central mediator in TGF-$\beta$/SMAD signaling, which translocates from the cytoplasm to the nucleus to regulate gene expression. The importance of BMP signaling in the regulation of hepcidin expression is further supported by studies in mice with liver-specific disruption of SMAD4, which show decreased hepcidin expression and severe iron accumulation in the liver as well as other organs (28).

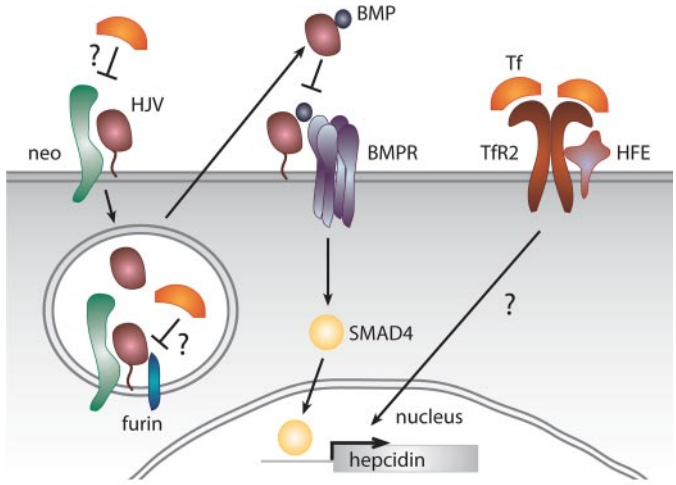


FIGURE 1. **Model of hepcidin regulation by iron.** Central to this model is that hepcidin transcription is regulated by HJV, which acts as a co-receptor for BMP. Upon binding to the BMP receptor (*BMPR*), a signaling cascade is initiated resulting in the translocation of SMAD4 to the nucleus, where it stimulates hepcidin transcription. The binding of HJV to neogenin (*neo*) is necessary for the release of HJV from cells. The release is dependent on cleavage by the protease furin. Where in the cell this occurs remains to be determined. In addition, cleavage of HJV is inhibited by Tf. Soluble HJV inhibits BMP-mediated signaling. Mutations in TfR2, HFE, or Tf result in a decreased level of hepcidin mRNA. Tf stabilizes TfR2. The mechanism by which the Tf-TfR2-HFE complex affects hepcidin transcription is unknown.

HJV is a glycosylphosphatidylinositol-linked protein that undergoes regulated release from cells. Its release depends on its interaction with neogenin, a membrane protein widely expressed in different tissues, including liver and muscle, but not on BMP signaling (29). Release is negatively regulated by iron-loaded Tf and possibly non-Tf iron (29–31). Recent reports implicate the protease furin in the cleavage and secretion of HJV (30, 31), but the details of how it is controlled are controversial. *In vitro* and *in vivo* studies of soluble HJV suggest that the regulation of HJV release in response to body iron loading plays a key role in signaling (27). In this paradigm, HJV release from both skeletal muscle and liver is negatively regulated by body iron status to modulate the soluble HJV levels in serum (29). Soluble HJV competes with hepatocyte membrane HJV for the limited BMP local supply and negatively regulates hepatic hepcidin expression (Fig. 1). Interesting, BMP9, a cytokine highly expressed in liver non-parenchymal cells, and BMP7 robustly induce hepatic hepcidin expression but do not bind HJV. Therefore, other mechanisms by which HJV regulates hepatic hepcidin expression are likely.

*TfR2*—The recently identified TfR2 is a second, distinct TfR and is most likely responsible for the previously reported non-TfR1-mediated uptake of Tf into the liver. TfR2 is postulated to

be involved in the sensing of iron-loaded Tf levels in the blood. Mutations in TfR2 are associated with a recessive rare form of HH (32). The observation that *Tfr2*$^{-/-}$ mice have similar iron overload as disease-causing mutations confirms that a loss of function in the *TfR2* gene causes this form of HH (33). Like TfR1, TfR2 is a type II membrane glycoprotein with a large C-terminal ectodomain and small N-terminal cytoplasmic domain (18, 34). TfR2 shares 45% amino acid sequence identity with TfR1 in the extracellular region. Clear differences exist between the two TfRs. The affinity of TfR2 for iron-loaded Tf is ~30-fold lower than that of TfR1 (18, 35). Although both receptors have internalization motifs, there are no sequence similarities in their cytoplasmic domains. TfR2 is much less stable than TfR1, allowing changes in TfR2 over a shorter time period (36, 37).

TfR2 is regulated at the level of protein degradation by a novel mechanism. It is stabilized by diferric Tf *in vitro* and *in vivo* (36, 37). TfR2 increases in a time- and dose-dependent manner after addition of diferric Tf to the culture medium. The response to diferric Tf appears to be hepatocyte-specific. Non-hepatic cell lines that either endogenously express TfR2 such as K562 cells or are transfected with a plasmid encoding TfR2 do not respond to Tf (36, 37). Real-time quantitative reverse transcription-PCR analysis shows that TfR2 mRNA levels do not change in cells treated with diferric Tf (36). Rather, the Tf-mediated up-regulation of TfR2 is due to an increase in the half-life of the protein (36). The binding of Tf to TfR2 appears to be responsible for these effects. Unlike wild-type TfR2, the level of a mutant form of TfR2 that does not detectably bind Tf does not increase in response to diferric Tf (38). These results support a role for TfR2 in monitoring iron levels by sensing changes in the concentration of iron-loaded Tf.

Animal studies are consistent with these observations. Rats fed an iron-deficient diet have lower TfR2 levels and Tf saturations than rats fed a high iron diet (37). TfR2 and Tf saturation are higher in HFE knock-out mice compared with normal littermates (37). TfR2 is also lower in the hypotransferrinemic mouse, supporting the role of diferric Tf in the stabilization of TfR2 (37). Because both tissue culture and animal studies show that TfR2 levels are sensitive to Tf over physiological ranges of Tf saturation, we and others hypothesize that TfR2 is the sensor for body iron levels. In keeping with this observation is a report showing that mice with a disease-causing mutation in TfR2 have decreased hepcidin mRNA levels (39).

*HFE*—The most prevalent form of HH is the autosomal recessive disease caused by a mutation in HFE (40). In the United States, the carrier frequency of this mutation is ~1 in 9 for individuals of Northern European heritage, making it the most common potentially lethal inherited disease in this population. The penetrance of the gene is still debated, with estimates ranging from 1:400 to 1:10,000 individuals having the clinical disease (41). The penetrance in men is much higher than in women. The mutation in 83% of HH is a single base G-to-A transition in nucleotide 845 that converts Cys to Tyr (42). The HFE mutant fails to associate with $\beta_2$-microglobulin and is not transported to the plasma membrane (43). Both *Hfe*$^{-/-}$ mice and $\beta_2$-microglobulin knock-out mice are similarly iron-overloaded (44, 45), confirming that the HFE mutant has decreased function.

Several recent findings point toward a mechanism by which HFE regulates hepcidin production in the liver. First is the discovery that HFE associates with TfR2 (46, 47). Mutations in either TfR2 or HFE result in a 2-fold decrease in hepcidin mRNA for a given iron load. A decrease in hepcidin would increase FPN levels, which would account for the increased uptake of iron by the intestine. These results implicate the HFE-TfR2 complex in the sensing of body iron levels (Fig. 1). The second finding that HFE increases TfR2 levels is consistent with a signaling role for TfR2 (48). Higher amounts of TfR2 would be able to signal to a greater extent. The most recent finding using mutant forms of TfR1 that either cannot bind Tf but can bind HFE or can bind Tf but not HFE shows that TfR1 serves as a reservoir of HFE and that iron-loaded Tf modulates the release of HFE from TfR1. A TfR1 mutant that fails to bind HFE results in the up-regulation of hepcidin transcription (47). It was speculated that the release of HFE from TfR1 by Tf allows HFE to bind to TfR2. This model is very appealing in that it gives a function to HFE, which is expressed mainly in hepatocytes (49), and explains the recent finding that hepatocyte-specific expression of HFE is able to prevent iron loading in the livers of *Hfe*$^{-/-}$ mice (50) but intestine-specific expression of HFE is not (51).

## Other Regulators of Hepcidin

*Erythroid Factor*—The sensors for communicating body iron stores and erythropoietic state are only beginning to be understood. Early physiological studies demonstrated that soluble factor(s) in the blood are involved. Iron-loaded Tf, ferritin, serum TfR1 generated from the proteolytic cleavage of full-length transmembrane TfR1, and hepcidin have been proposed as candidate factors (52–58). Tf, ferritin, and serum TfR1 are found in serum and fluctuate with iron status of the individual. The amount of serum ferritin increases in iron-overloaded individuals. There are two notable exceptions to the correlation of these proteins with iron stores within an organism. Mice lacking or having very low levels of Tf suffer from iron overload (59), implying a possible role for Tf in the sensing of iron stores. This finding fits with the hypothesis that intestinal iron absorption is regulated according to Tf saturation levels and that, in the absence of Tf, dietary iron is transported into the blood without regulation. The second exception is hyperferritinemic individuals. A mutation in the stem-loop structure of L-ferritin results in unregulated ferritin synthesis, leading to high serum ferritin levels and cataracts (60, 61). Notably, these people do not suffer from iron overload (61). Thus, serum ferritin levels are not likely to be a key part of the sensing mechanism for iron absorption. Serum TfR1 fluctuates with erythropoietic activity and iron status of the organism (52). Approximately 80% of serum TfR1 is generated by the maturation of erythroid cells (62). One argument against a role for serum TfR1 as an erythroid regulatory factor is that it is generated after cells no longer need iron for hemoglobin biosynthesis.

A recent study implicates the growth differentiation factor GDF15 as the candidate for the long-seeking erythroid factor (63). GDF15 is a family member of the TGF-$\beta$ superfamily and

is secreted by erythroid precursors. In patients with β-thalassemia, the defective erythroid expansion is correlated with an elevated level of serum GDF15. *In vitro* studies demonstrated a suppressive effect of GDF15 on hepcidin expression. These findings are consistent with the previous assumption that erythropoiesis is positively linked to intestinal iron absorption and storage iron mobilization and that the erythroid factor dominantly suppresses hepcidin expression despite iron overload.

*Hypoxia*—Hypoxia is another suppressor of hepatic hepcidin expression independent of body iron levels. The hypoxia-inducible transcription factors (HIFs) play a vital role in this process. The protein levels of HIF are negatively regulated by iron and oxygen. In the presence of oxygen, its subunits are modified by iron-dependent prolyl hydroxylases. The modified HIF interacts with the von Hippel-Lindau factor and is subsequently targeted for degradation through the ubiquitin/proteasome pathway. Under hypoxia or following iron chelation, the prolyl hydroxylase activity is inhibited, resulting in the accumulation and translocation of HIF into the nucleus. HIF binding to the promoter of hepcidin leads to the suppression of hepcidin expression in hepatocytes (64) and increased iron uptake to meet the erythropoietic demand.

*Inflammation*—Inflammation is a dominant and robust inducer of hepcidin gene transcription regardless of body iron levels. Interleukin-6 and possibly other inflammatory cytokines are the major players in this process. The binding of STAT3 (signal transducer and activator of transcription-3) to the promoter of hepcidin activates transcription (10). The finding that SMAD4-deficient hepatocytes from mice with a liver-specific SMAD4 deficiency have an abrogated hepcidin response to interleukin-6 (28) indicates that the inflammation-induced hepcidin expression acts through the TGF-β/SMAD4 signaling pathway.

## Summary

Iron homeostasis occurs largely through the regulation of hepatic hepcidin expression. The BMP/SMAD signaling pathway appears to play a pivotal role in this process. Functional disruption of the body iron-sensing proteins (HJV, TfR2, and HFE) constitutes the major cause of HH. How these proteins coordinately sense body iron levels, modulate BMP/SMAD signaling, and regulate hepcidin expression remain to be determined.

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